Characterization of the Reactive Sulfhydryl Groups in Carbamyl Phosphate Synthetase of *Escherichia coli**

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ABSTRACT: At least three different SH groups have been identified in carbamyl phosphate synthetase from *Escherichia coli* B by showing that they are available for reaction with either of the two SH reagents *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid) under different conditions and that different effects on catalytic activity are observed as a result of these reactions with each of the different SH groups. One SH group reacts under all conditions studied with no effect on the catalytic activities of the enzyme; this is the only SH group which reacts when ornithine is present. A second SH group reacts when ATP–$MgCl_2$ and bicarbonate are also present; the reaction is accompanied by an increase in the apparent K_m for glutamine and a 50% decrease in the carbamyl phosphate synthetase and ATP synthesis activities of the enzyme. A third SH group can be titrated when higher concentrations of the SH reagents are used. UMP is not required for this reaction, but increases the rate of reaction, while ornithine and ATP–$MgCl_2$ plus bicarbonate prevent the reaction; the reaction is accompanied by virtually complete loss of the carbamyl phosphate synthetase activity, but 100% activation of the ATP synthesis reaction. Stoichiometric studies with both of these reagents suggest that there is one of each of these different SH groups present per enzyme molecule and that the molecular weight of the enzyme is in the range of 170,000–200,000.

Carbamyl phosphate synthetase from *Escherichia coli* B catalyzes the following reaction (Anderson and Meister, 1965)

$$2ATP + HCO_3^- + \text{L-glutamine} + H_2O \xrightarrow{K^+,\ Mg^{2+}} 2ADP + P_i + \text{carbamyl phosphate} + \text{L-glutamate}$$

The catalytic activity of this enzyme can be inhibited by reaction with *N*-ethylmaleimide (NEM)[1] (Anderson and Marvin, 1970). The presence of ornithine, a positive allosteric effector (Anderson and Marvin, 1968; Pierard, 1966), prevents this reaction, whereas the rate of inhibition is markedly increased when the substrates ATP–$MgCl_2$ and bicarbonate are present; this marked effect of ATP–$MgCl_2$ and bicarbonate is virtually eliminated when UMP, a negative allosteric effector (Anderson and Meister, 1966b), is also present, but is facilitated when ornithine is present (Anderson and Marvin, 1970). A scheme similar to that shown in Figure 12 was previously suggested to account for these and other observations; it was assumed that the reaction with NEM was with SH group(s) on the enzyme and that the availability of these SH group(s) for reaction with NEM varies with the different conformational states of the enzyme which are presumed to exist in the presence of the different ligands (Anderson and Marvin, 1970).

In this paper evidence is presented which indicates that at least three different SH groups are involved in the reaction with NEM, that these SH groups will also react with DTNB, and that the availability of each of these SH groups for reaction with NEM or DTNB is dependent on the presence or absence of different ligands so that they can be titrated individually. In addition to the overall reaction above (synthetase activity) carbamyl phosphate synthetase also catalyzes a bicarbonate-dependent hydrolysis of ATP (ATPase activity) and the synthesis of ATP from ADP and carbamyl phosphate (ATP synthesis activity) (Anderson and Meister, 1966a); the effects of the reactions of NEM or DTNB on the catalytic activities are described.

Materials and Methods

Carbamyl phosphate synthetase was isolated from *E. coli* B as described by Anderson *et al.* (1970). *E. coli* cell paste (washed, 3/4 log phase, grown on enriched medium) was purchased from Grain Processing Corp.

Ornithine, UMP, Na_2ATP, glutamine, DTNB, cysteine, and NEM were purchased from Sigma Chemical Co. [^{14}C]-$NaHCO_3$ was obtained from New England Nuclear Corp.

Synthetase activity was routinely determined by measuring the rate of ADP formation; the aliquot containing enzyme was added to a reaction mixture containing ATP (6 μmoles), $MgCl_2$ (6 μmoles), glutamine (3 μmoles), $NaHCO_3$ (3 μmoles), KCl (30 μmoles), and Tris-HCl buffer (30 μmoles, pH 8.2) at 37° to give a final volume of 0.3 ml; the ADP formed after 10 min at 37° was determined by coupling with lactate dehydrogenase and pyruvate kinase as previously described (Anderson and Meister, 1966a). ATPase activity was determined in the same way except the reaction mixture contained ATP (3 μmoles), $MgCl_2$ (3 μmoles), $NaHCO_3$ (3 μmoles), and potassium phosphate buffer (30 μmoles, pH 7.8) (Anderson and Meister, 1966a). In some cases synthetase activity was also determined by measuring the rate of formation of [^{14}C]carbamyl phosphate from [^{14}C]$NaHCO_3$ as previously described (Anderson and Meister, 1965). ATP synthesis activity was routinely determined by measuring the rate of formation of

* From the Department of Chemistry, Southern Illinois University, Carbondale, Illinois 62901. *Received July 20, 1971.* This work was supported in part by U. S. Public Health Service Grant AM11443. A preliminary account of this work has appeared (Anderson *et al.*, 1970).

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[1] Abbreviations used are: NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); abbreviations for the various enzyme derivatives are described in the text.

ATP; the aliquot containing enzyme was added to a reaction mixture containing ADP (6 μmoles), $MgCl_2$ (6 μmoles), lithium carbamyl phosphate (3 μmoles), and potassium phosphate buffer (30 μmoles, pH 7.8) to give a final volume of 0.3 ml; the ATP formed after 10 min at 37° was determined by coupling with hexokinase and glucose 6-phosphate dehydrogenase as previously described (Anderson and Meister, 1966a).

[^{14}C]NEM was obtained from Schwarz BioResearch. The concentration and radiochemical purity of this material were established by measuring the absorbance at 300 mμ (Benesch and Benesch, 1962) followed by quantitative reaction with excess cysteine and measurement of the decrease in absorbance at 300 mμ; an aliquot of this reaction mixture was then subjected to paper chromatography and the per cent of the radioactivity in the ninhydrin-positive spot corresponding to the expected reaction product, *S*-cysteino(*N*-ethyl)succinimide, determined. The value obtained was 85% which was in close agreement with the suppliers' data.

The incorporation of radioactivity into protein when [^{14}C]-NEM was reacted with carbamyl phosphate synthetase was measured as follows. The reaction was stopped and the protein precipitated by adding the aliquot of the reaction mixture to 0.5 ml of a solution containing 5% trichloroacetic acid and unlabeled NEM (10 mM). The precipitated protein was washed three times with 3 ml of 5% trichloroacetic acid and twice with 4 ml of ethanol. After the ethanol was removed by evaporation *in vacuo* 1 ml of a solution containing Tris-HCl buffer (0.2 M, pH 8.3), $CaCl_2$ (15 mM), and Pronase (1 mg/ml; a protease from *Streptomyces griseus* purchased from Calbiochem) was added; after the protein was solubilized due to hydrolysis by the protease 0.2 ml of 0.25 M $BaCl_2$ was added and the sample dried on a planchet for counting with a Nuclear-Chicago gas-flow counter.

Identification of the Site of Reaction of [^{14}C]NEM with Carbamyl Phosphate Synthetase. The site of reaction of [^{14}C]NEM with carbamyl phosphate synthetase was identified as SH groups of cysteine residues as follows. The protein in that portion of each of the reaction mixtures described in Figures 2 and 6 which remained after termination of the experiment was precipitated with 5% trichloroacetic acid containing unlabeled NEM (10 mM). The precipitated protein was washed with 5% trichloroacetic acid, acetone, and finally with ethanol, and then hydrolyzed for 48 hr with 6 N HCl at 110° (Moore and Stein, 1963); a sample containing the product of the reaction between [^{14}C]NEM and excess cysteine and an amount of unreacted enzyme equivalent to the quantity in the other samples was also subjected to hydrolysis under the same conditions and served as a control and reference. Aliquots of each hydrolysate were then subjected to descending paper chromatography in two different solvent systems: butanol–acetic acid–water (12:3:5, v/v) and phenol–water–NH_4OH (16:14:1, v/v); *S*-cysteino(*N*-ethyl)succinimide was added to the samples as a carrier. In both solvent systems the radioactivity in all three samples migrated with the *S*-cysteino(*N*-ethyl)succinimide which was located by reaction with ninhydrin.

A Beckman Model DBG spectrophotometer with a Beckman recorder was used for the titration studies with DTNB. Enzyme concentration was measured by its absorbance at 280 mμ (Anderson and Marvin, 1970).

Results

Amino Acid Analysis. The results of amino acid analysis of carbamyl phosphate synthetase are shown in Table I. The calculated total number of half-cystine residues is nearly equal to the number of cysteine residues as determined by titration of the free SH groups with DTNB, suggesting that few if any disulfide bonds exist in the enzyme. As will be shown in the following sections, only one of the SH groups reacts readily with DTNB or NEM in the absence of substrates or allosteric effectors.

TABLE I: Amino Acid Composition of Carbamyl Phosphate Synthetase.

Amino Acid	Residues/Molecule[a] Calcd	Nearest Integer
Lysine	96.2	96
Histidine	35.7	36
Arginine	101.7	102
Aspartic acid	182.1	182
Threonine	106.5	107
Serine	80.4	80
Glutamic acid	224.7	225
Proline	76.3	76
Glycine	147.7	148
Alanine	194.5	195
Valine	134.0	134
Methionine	45.3	45
Isoleucine	109.2	109
Leucine	143.0	143
Tyrosine	53.6	54
Phenylalanine	70.8	71
Tryptophan[b]	15.1	15
Half-cystine[c]	21.0	21
Cysteine[d]	18.8	19

[a] Amino acid analysis was carried out as described by Moore and Stein (1963) using a Beckman 120C amino acid analyzer. The values reported are those determined by extrapolation to zero time (threonine and serine) or averaging the values of samples hydrolyzed for 24, 48, and 70 hr, respectively. A value of 200,000 was assumed for the molecular weight. The amide composition was not determined. [b] Determined by the method of Edolhoch (1967). [c] Determined by performic acid oxidation (Bailey, 1967). [d] The total free SH groups (cysteine) were determined by titration with DTNB (2.5 mM) in potassium phosphate buffer (0.1 M, pH 7.0) containing guanidine hydrochloride (6.0 M) and enzyme (0.17–0.6 mg/ml) (Ellman, 1959).

Reaction of Carbamyl Phosphate Synthetase with NEM in the Presence of Ornithine. The incorporation of radioactivity into carbamyl phosphate synthetase with time when the enzyme is incubated with [^{14}C]NEM in the presence of ornithine is shown in Figure 1. The reaction is apparently complete after 30 min; calculations from the data in Figure 1 show that at 30 min about 1 mole of [^{14}C]NEM has become incorporated per 200,000 g of enzyme. As shown in Figure 1 and Table II, and as expected from previous studies (Anderson and Marvin, 1970), there is no loss in catalytic activity as a result of this reaction. The stoichiometry of this reaction would suggest that a single SH group is involved, since previous studies (Anderson and Marvin, 1968, 1970) indicate a maximum molecular weight of about 200,000 for carbamyl phosphate

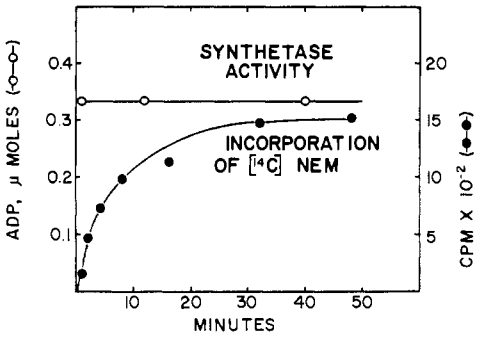

FIGURE 1: Reaction of [^{14}C]NEM with carbamyl phosphate synthetase in the presence of ornithine. The reaction mixture contained enzyme (10.5 mg/ml), potassium phosphate buffer (0.13 M, pH 7.5), EDTA (0.3 mM), [^{14}C]NEM (0.38 mM, 130, 240 cpm), and ornithine (16.6 mM) at 17° in a final volume of 0.3 ml. At the indicated times a 5-μl aliquot was removed and added to 0.5 ml of a solution containing potassium phosphate buffer (0.18 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction; the synthetase activity is expressed as the micromoles of ADP formed when a 50-μl aliquot of each of these solutions was subsequently assayed for 10 min as described in the text. At the indicated times a 25-μl aliquot was also removed from the reaction mixture and the counts per minute of [^{14}C]NEM bound to the protein in each of the aliquots determined as described in the text; the quantity of [^{14}C]NEM bound to the protein is expressed as counts per minute of [^{14}C]NEM bound to the protein in the 25-μl aliquot.

synthetase. As demonstrated by the results presented below, ornithine is apparently not required for this reaction, but simply prevents reaction with other SH groups. The product of this reaction will be referred to as CPSase–[NEM]$_1$.

Reaction of Carbamyl Phosphate Synthetase with NEM in the Presence of ATP–$MgCl_2$ and Bicarbonate. The results of an experiment similar to that described above, but carried out in the presence of ATP–$MgCl_2$ and bicarbonate, are described in Figure 2. Calculations from the data in Figure 2 show that in this case 2 moles of [^{14}C]NEM are incorporated per 200,000

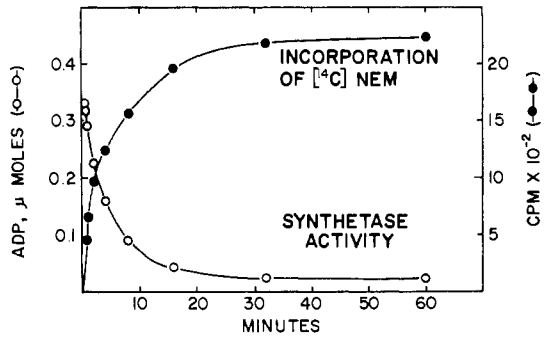

FIGURE 2: Reaction of [^{14}C]NEM with carbamyl phosphate synthetase in the presence of ATP–$MgCl_2$ and bicarbonate. The reaction mixture contained enzyme (10.5 mg/ml), potassium phosphte buffer (0.13 M, pH 7.5), EDTA (0.3 mM), and $NaHCO_3$ (16.7 mM) at 17° in a final volume of 0.45 ml. At the indicated times a 5-μl aliquot was removed and added to 0.5 ml of a solution containing potassuim phosphate buffer (0.18 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction; the synthetase activity is expressed as the μmoles of ADP formed when a 50-μl aliquot of each of these solutions was subsequently assayed for 10 min as described in the text. At the indicated times a 25-μl aliquot was also removed from the reaction mixture and the counts per minute of [^{14}C]NEM bound to the protein in the aliquots determined as described in the text; the quantity of [^{14}C]NEM bound to the protein is expressed as counts per minute of [^{14}C]NEM bound to the protein in the 25-μl aliquot.

TABLE II: Comparison of the Specific Activities of Derivatives of Carbamyl Phosphate Synthetase Obtained by Reaction with NEM.[a]

Enzyme Derivative and Additions	Specific Activity		
	Synthetase	ATP Synthesis	ATPase
Control			
No addition	104	16	11
Ornithine	154	19	
UMP	11	<1	
CPSase–[NEM]$_1$			
No addition	103	15	11
Ornithine	148	19	
UMP	11	<1	
CPSase–[NEM]$_2$			
No addition	18	8	3
Ornithine	22	9	
UMP	1	<1	

[a] CPSase–[NEM]$_1$ and CPS–[NEM]$_2$ were prepared as described in Figures 10 and 3, respectively. In addition to enzyme, the reaction mixtures used for assay of the different activities were as follows. Synthetase: ATP (10 mM), $MgCl_2$ (10 mM), $NaHCO_3$ (10 mM), glutamine (10 mM), potassium phosphate buffer (0.1 M, pH 7.8), and where indicated ornithine (3.3 mM) or UMP (0.4 mM) in a final volume of 0.3 ml. ATP synthesis: ADP (10 mM), $MgCl_2$ (10 mM), lithium carbamyl phosphate (10 mM), potassium phosphate buffer (0.13 M, pH 7.8), and where indicated ornithine (10 mM) or UMP (1.3 mM) in a final volume of 0.3 ml. ATPase: ATP (10 mM), $MgCl_2$ (10 mM), $NaHCO_3$ (10 mM), and potassium phosphate buffer (0.1 M, pH 7.8) in a final volume of 0.3 ml. The μmoles of product (ADP or ATP) formed after incubation for 10 min at 37° were determined as described in the text. The specific activity indicates micromoles of product per hour per milligram of enzyme.

g of the enzyme during the course of the reaction, indicating that due to the presence of ATP–$MgCl_2$ and bicarbonate one additional SH group is made available which reacts readily with [^{14}C]NEM; the other SH group is presumably the same as that which reacts with NEM in the presence of ornithine as described above (also, see results of studies with DTNB). The product of this reaction will be referred to as CPSase–[NEM]$_2$.

As shown in Figures 2 and 3 and in Table II, this reaction is accompanied by a 90 and 50% decrease in the synthetase and ATP synthesis activities of carbamyl phosphate synthetase, respectively, as well as a substantial decrease in the ATPase activity (Table II). However, the observed maximum loss in synthetase activity can be reduced to about 50% if the concentration of glutamine in the standard assay solution is increased tenfold (see Figure 5). The effect of glutamine concentration on the synthetase activity of CPSase–[NEM]$_2$ is shown in Figure 4 where the data are plotted by the method of Lineweaver and Burk (1934); the apparent K_m value for glutamine is 0.02 M, a 50-fold increase from the value of 3.8×10^{-4} M for the native enzyme (Anderson and Meister, 1966a). Experiments similar to that described in Figures 3 and 4 have also been carried out in which the synthetase activity was

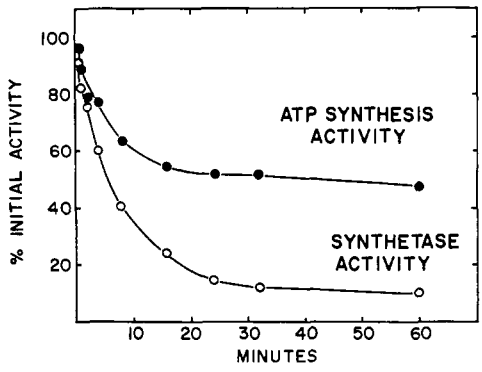

FIGURE 3: Inhibition of synthetase and ATP synthesis activities by reaction of carbamyl phosphate synthetase with NEM in the presence of ATP-$MgCl_2$ and bicarbonate. The reaction mixture contained potassium phosphate buffer (0.17 M, pH 7.5), EDTA (0.4 mM), NEM (0.8 mM), ATP (16.5 mM), $MgCl_2$ (16.5 mM), $NaHCO_3$ (16.5 mM), and enzyme (16 mg/ml) at 17° in a final volume of 2.42 ml. At the indicated times a 20-μl aliquot was removed and added to 0.5 ml of a solution containing potassium phosphate buffer (0.1 M) and ornithine (10 mM) at 4° to stop the reaction. The synthetase and ATP synthesis activities in 10- and 100-μl aliquots, respectively, of each of these solutions were determined by measuring the micromoles of ADP and ATP, respectively, formed in 10 min as described in the text; the initial activities were 0.53 μmole of ADP and 0.13 μmole of ATP, respectively. CPSase-$[NEM]_2$ was separated from other components of the reaction mixture after the experiment was completed (60 min) by gel filtration on Sephadex G-50 in potassium phosphate buffer (0.2 M, pH 7.5).

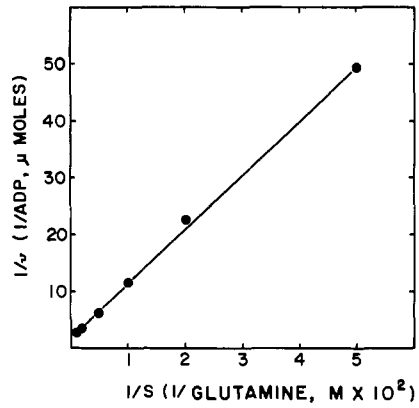

FIGURE 4: Effect of glutamine concentration on the synthetase activity of CPSase-$[NEM]_2$. The reaction mixtures contained ATP (20 mM), $MgCl_2$ (20 mM), NaHCO (10 mM), Tris-HCl buffer (100 mM, pH 8.1), CPSase-$[NEM]_2$ (0.017 mg), and glutamine as indicated in a final volume of 0.3 ml. The ADP formed after 10 min at 37° was determined as described in the text.

assayed by measuring the rate of carbamyl phosphate formation instead of ADP; identical results were obtained (see Figure 5); identical results are also obtained, as would be expected (Anderson and Marvin, 1970), when the reaction is carried out with ornithine also present.

The effects of ornithine and UMP on the synthetase activity of CPSase-$[NEM]_2$ as a function of ATP-$MgCl_2$ concentration are shown in Figure 5; these effects are virtually identical with those obtained with the native enzyme, indicating that the "regulatory" effects of ornithine and UMP on the enzyme activity are not significantly altered by reaction of these SH groups with NEM; in addition, sucrose density gradient studies have shown that the properties of CPSase-$[NEM]_2$ are identical with the native enzyme's with respect to the previously reported variation of the sedimentation constant with enzyme concentration and the effect of ornithine and UMP on the monomer-oligomer equilibrium (Anderson and Marvin, 1970).

Further Reaction of CPSase-[NEM]$_2$ with NEM. In the presence of UMP and somewhat higher concentrations of [^{14}C]NEM at least one more SH group will react with the [^{14}C]NEM (Figure 6). During the course of this reaction the synthetase activity is almost completely abolished, but of considerably more interest is the fact that the ATP synthesis activity increases almost twofold to a level nearly the same as that of the native enzyme. The incorporation of radioactivity continues after the effects on catalytic activity have ceased, indicating that additional SH groups are reacting slowly with the [^{14}C]NEM. Extrapolation of the slope representing the rate of incorporation after 30 min back to zero time yields a value of 1.4 SH groups/200,000 g of enzyme; although this value is subject to considerable error, it would suggest that only one SH group per enzyme molecule is involved in the observed changes in catalytic activity. It should be pointed out that UMP is not required for this reaction, but simply increases the rate when high enzyme concentrations are used (see Figure 7), possibly by maintaining the enzyme in the conformation designated monomer I in the scheme illustrated in Figure 12; in the absence of UMP the equilibrium would be partly shifted to monomer II and oligomer under the conditions used (Anderson and Marvin, 1970).

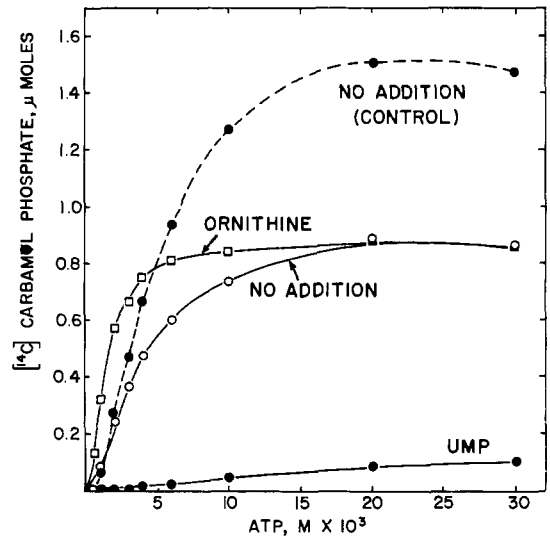

FIGURE 5: Effect of ornithine and UMP on the rate of synthesis of [^{14}C]carbamyl phosphate by CPSase-$[NEM]_2$ as a function of ATP concentration. The reaction mixtures contained $MgCl_2$ in concentrations that were equimolar with ATP, ATP as indicated, [^{14}C]$NaHCO_3$ (10 mM, 164,000 cpm), glutamine (100 mM), and CPSase-$[NEM]_2$ (0.03 mg) in a final volume of 1.0 ml. Where indicated the reaction mixtures also contained ornithine (10 mM) or UMP (10 mM). The [^{14}C]carbamyl phosphate synthesized after incubation for 10 min at 37° was determined as described in the text. CPSase-$[NEM]_2$ was prepared just before use by incubating carbamyl phosphate synthetase (1.2 mg) in a reaction mixture (0.3-ml final volume) containing ATP (13 mM), $MgCl_2$ (13 mM), $NaHCO_3$ (13 mM), NEM (0.53 mM), and potassium phosphate buffer (0.16 M, pH 7.8) for 30 min at 17° and then diluting with 4.2 ml of 0.2 M potassium phosphate buffer, pH 7.8 at 4°; an 0.1-ml aliquot of this solution was used in each of the above reaction mixtures. The control (dashed line) represents enzyme prepared in exactly the same way except NEM was omitted.

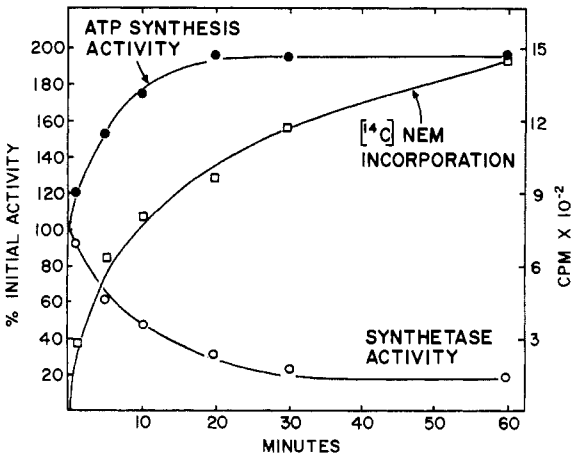

FIGURE 6: Reaction of [^{14}C]NEM with CPSase–[NEM]$_2$. The reaction mixture contained potassium phosphate buffer (0.1 M, pH 7.8), EDTA (0.1 mM), [^{14}C]NEM (1 mM, 282,000 cpm), UMP (1.2 mM), and CPSase–[NEM]$_2$ (4.0 mg/ml) at 17° in a final volume of 1.2 ml. At the indicated times a 25-μl aliquot was removed and added to 0.2 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction. The synthetase and ATP synthesis activities in 100-μl aliquots of each of these solutions were determined by measuring the micromoles of ADP and ATP, respectively, formed in 10 min in the presence of ornithine (20 mM) as described in the text; the initial activities for each of these two reactions were 0.28 and 0.06 μmole of ADP and ATP, respectively. At the indicated times a 25-μl aliquot was also removed from the reaction mixture and the counts per minute of [^{14}C]NEM bound to the protein in the aliquots determined as described in the text. CPSase–[NEM]$_2$ was prepared as described in Figure 3.

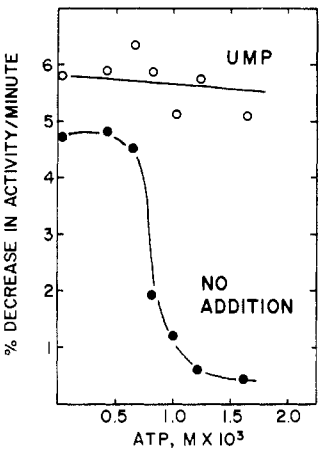

FIGURE 8: Effect of ATP on the rate of inhibition of CPSase–[NEM]$_2$ by NEM. Reaction mixtures containing potassium phosphate buffer (0.17 M, pH 7.8), EDTA (0.4 mM), $NaHCO_3$ (10 mM), CPSase–[NEM]$_2$ (2 mg/ml), $MgCl_2$ in concentrations that were equimolar with ATP, ATP as indicated, and where indicated UMP (2 mM) were incubated with NEM (2.5 mM) at 17° in a final volume of 0.12 ml. At 1-min intervals for 5 min a 10-μl aliquot was removed from each reaction mixture and added to 0.2 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction. The synthetase activity in 100-μl aliquots of each of these solutions was determined by measuring the micromoles of ADP formed in 10 min as described in the text. A linear decrease in activity with time was obtained for each reaction mixture; the rate of decrease in activity is expressed as per cent decrease in activity per minute. CPS–[NEM]$_2$ was prepared as described in Figure 3.

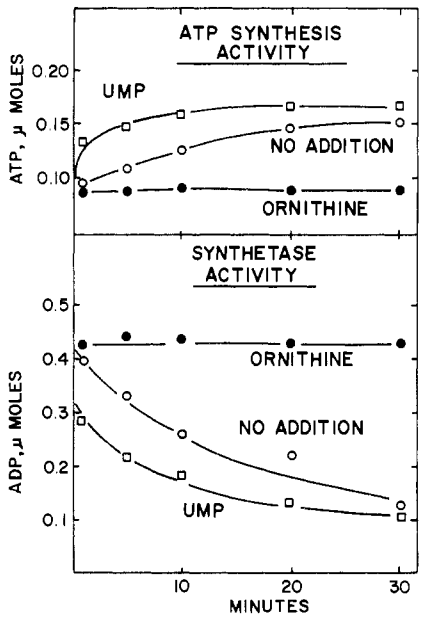

FIGURE 7: Effect of ornithine and UMP on the rate of inhibition of the synthetase and ATP synthesis activities of CPSase–[NEM]$_2$ by NEM. The reaction mixtures contained potassium phosphate buffer (0.1 M, pH 7.8), NEM (1 mM), CPSase–[NEM]$_2$ (3.5 mg/ml), and where indicated ornithine (20 mM) or UMP (1 mM) at 17° in a final volume of 0.28 ml. At the indicated times a 50-μl aliquot was removed and added to 0.2 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction. The enyzme activities in 100-μl aliquots of each of these solutions were determined by measuring the micromoles of ADP or ATP, respectively, formed in 10 min in the presence of ornithine (20 mM) as described in the text. CPSase–[NEM]$_2$ was prepared as described in Figure 3.

The effects of ornithine and UMP on the rate of reaction of CPSase–[NEM]$_2$ with NEM as measured by the rate of inhibition or activation of the two catalytic activities, respectively, are described in Figure 7; these results are similar to those reported previously for native enzyme (Anderson and Marvin, 1970).

The presence of ATP–$MgCl_2$ and bicarbonate will completely prevent this reaction. This is demonstrated in Figure 8 in which the effect of ATP–$MgCl_2$ concentration on the rate of inhibition of the synthetase activity is described; a sigmoidal-type curve similar to that describing the effect of ATP–$MgCl_2$ on the catalytic activity is obtained, and UMP prevents the protective effect of ATP–$MgCl_2$ which is consistent with the observation that UMP also prevents the effect of ATP–$MgCl_2$ in facilitating the reaction of NEM with the SH group involved in the formation of CPSase–[NEM]$_2$ (Anderson and Marvin, 1970) and that UMP inhibits catalytic activity (Anderson and Meister, 1966b).

Reaction of Carbamyl Phosphate Synthetase with DTNB. DTNB is a disulfide which will react specifically with SH groups in proteins and the stoichiometry of this reaction can be determined conveniently by simply measuring the product of the reaction spectrophotometrically at 412 mμ (Ellman, 1959). Reactions of carbamyl phosphate synthetase with DTNB similar to those carried out with NEM described above have been carried out with virtually the same results. Reaction in the presence of ATP–$MgCl_2$ and bicarbonate results in the titration of 2 SH groups/180,000 g of enzyme and loss of about 50 and 90% of the ATP synthesis and synthetase activities, respectively (Figure 9); neither activity is lost, however, if the enzyme assays are carried out in the presence of cysteine which presumably regenerates the free SH groups by disulfide interchange. By analogy with the reaction with NEM the

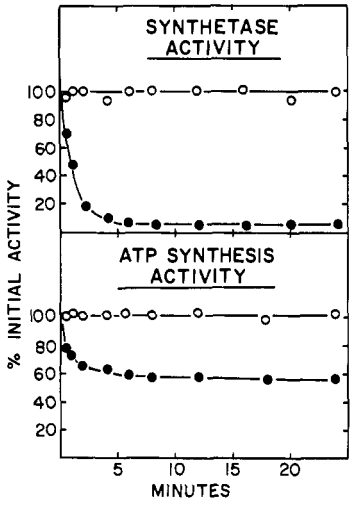

FIGURE 9: Inhibition of the synthetase and ATP synthesis activities of carbamyl phosphate synthetase by reaction with DTNB in the presence of ATP–$MgCl_2$ and bicarbonate. The reaction mixture contained potassium phosphate buffer (0.16 M, pH 7.5), DTNB (0.7 mM), ATP (10 mM), $MgCl_2$ (10 mM), $NaHCO_3$ (10 mM), and carbamyl phosphate synthetase (6 mg/ml) in a final volume of 0.3 ml at 17°. At the indicated times a 20-μl aliquot was removed and added to 0.8 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (0.02 M) at 4° to stop the reaction. The synthetase and ATP synthesis activities in 50- and 200-μl aliquots, respectively, of each of these solutions were determined by measuring the micromoles of ADP and ATP, respectively, formed in 10 min in the presence (open circles) or absence (closed circles) of cysteine (0.02 M) as described in the text; the initial activities were 0.42 μmole of ADP and 0.26 μmole of ATP, respectively.

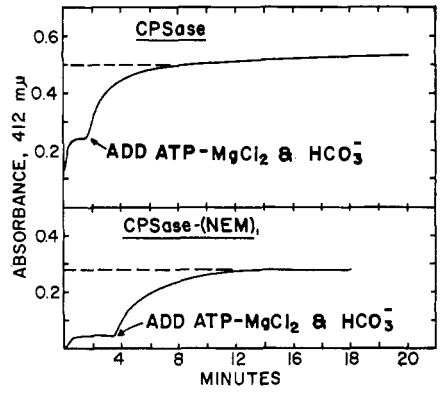

FIGURE 10: Sequential reaction of carbamyl phosphate synthetase with DTNB. Upper graph: a solution containing carbamyl phosphate synthetase (4.4 mg), potassium phosphate buffer (100 μmoles, pH 7.8), and ornithine (10 μmoles) in a volume of 1.22 ml was equilibrated to 17° in a cell of 10-mm path length in the sample compartment of the spectrophotometer (see text); the recorder was adjusted to zero absorbance using as a blank a matching cell in the reference compartment containing exactly the same solution as the sample cell except enzyme was absent. The reaction was initiated (zero time) by adding DTNB (0.5 μmole) in potassium phosphate buffer (10 μmoles, pH 7.8) in a volume of 0.1 ml to both the reference and sample cells and the increase in absorbance recorded with time. At the indicated time ATP, $MgCl_2$, and $NaHCO_3$ (5 μmoles of each) in a volume of 0.05 ml was added to each cell. Lower graph: the reaction was carried out in exactly the same way except CPSase-$[NEM]_1$ was used in place of native enzyme. CPSase-$[NEM]_1$ was prepared just before use by reacting carbamyl phosphate synthetase (18 mg) with NEM (0.4 mM) in 1.0 ml of a solution also containing potassium phosphate buffer (0.16 M, pH 7.5), ornithine (10 mM), and EDTA (0.4 mM) for 32 min at 17° (see Figure 1). The CPSase-$[NEM]_1$ was separated from the other reaction components by gel filtration in potassium phosphate buffer (0.2 M, pH 7.8).

product of this reaction will be referred to as CPSase-$[DTNB]_2$.

The sequential titration of the two SH groups which react with DTNB to yield CPSase-$[DTNB]_2$ is described in Figure 10. After titration of the SH group available for reaction with DTNB in the presence of ornithine a second SH group can be titrated by adding ATP–$MgCl_2$ and bicarbonate. Calculations of the stoichiometry of these reactions from these data yield values of 1 equiv of SH group reacted per 170,000 and 180,000 g of enzyme in the two consecutive reactions, respectively. Similar results are obtained when CPSase-$[NEM]_1$ is reacted with DTNB; the SH group which reacts with DTNB in the presence of ornithine is apparently the same SH group which reacts with NEM under the same conditions, since little reaction is observed in the presence of ornithine, but the addition of ATP–$MgCl_2$ and bicarbonate results in the titration of 1 equiv of SH group per 180,000 g of enzyme (Figure 10).

The further reaction of CPSase-$[DTNB]_2$ with excess DTNB is shown in Figure 11. The complete inhibition of synthetase activity is accompanied by a 100% increase in the ATP synthesis activity, as was observed when CPSase-$[NEM]_2$ was reacted with excess NEM. It is also apparent that at the higher concentrations of DTNB required to carry out this reaction in a reasonable time period the reaction continues after the changes in catalytic activity have ceased; extrapolation of the data to zero time yields a value of 1.2 SH groups/200,000 g of enzyme, suggesting that as in the case of the reaction with NEM one SH group is involved in the changes in catalytic activity.

Discussion

The three (or more) unique SH groups present in carbamyl phosphate synthetase as described in this study have been identified by showing that they are available for reaction with NEM or DTNB under different conditions and that different effects on catalytic activity are observed as a result of these reactions with each of the different SH groups. The results of these studies are consistent with and permit a further elaboration of the scheme proposed previously as a possible mechanism for explaining the results of studies on the allosteric effector- and ATP–$MgCl_2$ and bicarbonate-dependent inhibition by NEM and aggregation of carbamyl phosphate synthetase (Anderson and Marvin, 1970). According to this scheme the enzyme was considered to exist in at least three different conformational states, designated monomer I, monomer II, and monomer III, respectively. An extension of this scheme illustrating the disposition of the three different SH groups described in this paper is shown in Figure 12; the availability of an SH group for reaction with NEM or DTNB is indicated by its location outside the enclosures representing the different conformational states of the enzyme.

One SH group is apparently available under all conditions studied and this is the only SH group which reacts in the presence of ornithine (monomer II, Figure 12). The existence of a reactive SH group under these conditions was not previously observed because reaction of this SH group with NEM (or DTNB) does not affect the catalytic activity of the enzyme. The addition of ATP–$MgCl_2$ and bicarbonate results in the conversion of the enzyme to a different conformational state,

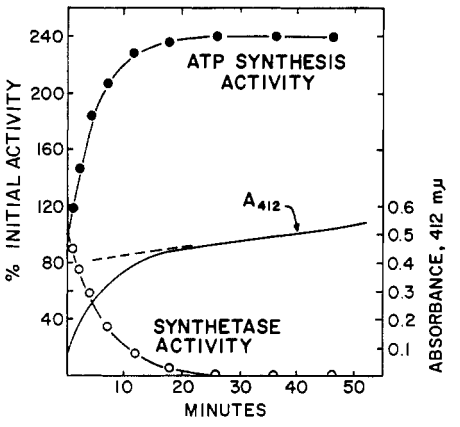

FIGURE 11: Reaction of CPSase-$[DTNB]_2$ with DTNB. The reaction was carried out essentially as described in Figure 10. The reaction mixture in the sample cell contained potassium phosphate buffer (0.1 M, pH 7.8), DTNB (1.8 mM), and CPSase-$[DTNB]_2$ (5.2 mg) in a final volume of 2.3 ml at 17°. The increase in absorbance at 412 mμ with time was recorded and at the indicated times a 50-μl aliquot was removed from the sample cell and added to 0.6 ml of a solution containing potassium phosphate buffer (0.1 M, pH 7.8) and ornithine (10 mM) at 4° to stop the reaction. The synthetase and ATP synthesis activities in 100-μl aliquots of each of these solutions were determined by measuring the micromoles of ADP and ATP formed in 10 min as described in the text; the initial activities were 0.16 μmole of ADP and 0.16 μmole of ATP, respectively. CPSase-$[DTNB]_2$ was prepared by incubating carbamyl phosphate synthetase with DTNB in the presence of ATP-$MgCl_2$ and bicarbonate as described in Figure 9 for 25 min and then separated from the other reaction components by gel filtration on Sephadex G-50 in potassium phosphate buffer (0.1 M, pH 7.8).

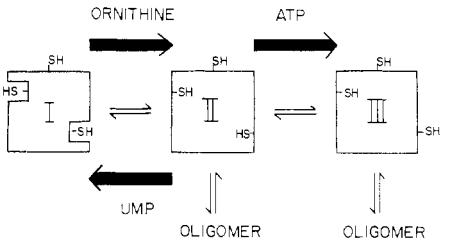

FIGURE 12: Scheme summarizing the conditions under which the SH groups described in the text are available for reaction with DTNB or NEM.

designated monomer III, which exposes an additional SH group which is susceptible to reaction with NEM or DTNB resulting in the decreases (but not elimination) in the catalytic activities described above. Although this SH group is not available for reaction with NEM or DTNB in the presence of ornithine (monomer II), the available data do not eliminate the possibility that this SH group is at least partially available for reaction when the enzyme exists in the conformational state designated monomer I (at least 3 equiv of SH groups per 200,000 g of enzyme can be titrated directly in the native enzyme in the presence of UMP with DTNB under conditions similar to those described in Figure 11 with the same effects on catalytic activity as those observed when CPSase-$(DTNB)_2$ is reacted with additional DTNB (Figure 11) (P. M. Anderson and R. E. Foley, 1970, unpublished data)).

In addition to these two SH groups, at least one additional SH group has been identified; the reaction of this SH group at an appreciable rate requires higher concentrations of DTNB or NEM and is facilitated by the presence of UMP (Figure 7) when the enzyme concentration is high (Anderson and Marvin, 1970). Consequently, this SH group is considered to be available for reaction only when the enzyme exists as monomer I; the fact that both ornithine and ATP-$MgCl_2$ and bicarbonate prevent this reaction is consistent with this conclusion. Since this SH group is apparently not as reactive as the other SH groups, it is depicted in the scheme in Figure 12 as being only partially exposed in monomer I, although the decreased reactivity could be explained by reasons other than steric hindrance of course. The most interesting and possibly significant observation made with respect to this SH group is the fact that its reaction with either NEM or DTNB results in (nearly) complete loss in synthetase activity, but activation of ATP synthesis activity. The implications of this observation with respect to the catalytic mechanism of carbamyl phosphate synthetase are not known at the present time. The ATP synthesis activity represents a partial reversal of the overall synthetase activity and is the reversal of the last step in the three-step mechanism previously proposed for the reaction pathway of carbamyl phosphate synthetase (Anderson and Meister, 1966a). The above results might implicate this SH group in an early step of the reaction pathway, its reaction with NEM or DTNB effectively inhibiting a step prior to that involved in the ATP synthesis activity.

The results of the studies on the stoichiometry of the reaction between NEM or DTNB and the different SH groups strongly suggest that there is only one of each of the different SH groups present per monomeric unit of enzyme and that the molecular weight of the monomeric unit is in the range of 170,000–200,000, which is in agreement with previous estimates made from sedimentation constants obtained from sucrose density gradient centrifugation studies (Anderson and Marvin, 1970). These results are consistent with recent studies which provide evidence that the monomeric unit of carbamyl phosphate synthetase is composed of two nonidentical subunits with molecular weights of about 140,000 and 48,000, respectively, and that each of the different SH groups is located in only one or the other of the two nonidentical subunits (Anderson *et al.*, 1970; P. M. Anderson and S. Matthews, 1970, unpublished data). In fact, other possible subunit structures would appear to be less likely if the one to one relationship between each different SH group and the enzyme molecule (monomeric unit) suggested by our findings is correct; a single polypeptide chain of such high molecular weight would be very unusual, and a structure composed of two or more identical subunits each containing these three different SH groups would require that the subunits occupy nonequivalent positions which would also be unusual (Klotz and Langerman, 1970).

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Rapid Kinetic Evidence for Adduct Formation between the Substrate Analog *p*-Nitroso-*N*,*N*-dimethylaniline and Reduced Nicotinamide–Adenine Dinucleotide During Enzymic Reduction*

M. F. Dunn† and Sidney A. Bernhard

ABSTRACT: In this study, the intense chromophore *p*-nitroso-*N*,*N*-dimethylaniline (NDMA) has been investigated as a substrate analog for equine liver alcohol dehydrogenase (LADH). The LADH-catalyzed reduction of NDMA by reduced nicotinamide–adenine dinucleotide (NADH) is accompanied by the formation of 1 mole of NAD^+/mole of NADH and NDMA consumed. The NDMA reduction product is (tentatively) assigned as *p*-*N*,*N*-dimethylaminophenylhydroxylamine on the basis of its uv spectrum, and on the forementioned stoichiometric relationships. At pH 8.75, the steady-state NDMA turnover number is 2.4-fold greater than the acetaldehyde turnover number. The Michaelis constant, $K_m^s = 7 \times 10^{-7}$ M, for NDMA is the smallest yet reported for an LADH substrate. The favorable spectral properties of this system allow the independent observation of both NDMA and NADH disappearance during reaction. The transient-state kinetic course of NDMA reduction by NADH has been investigated at pH 8.75 (0.05 M sodium pyrophosphate buffer), using a rapid-mixing stopped-flow spectrophotometer. Under the conditions of concentration [NDMA] > [E] > [NADH], the disappearance of NDMA occurs *via* two transformations which are clearly distinct in rate. NDMA disappears to the same apparent product in each transformation step in an amount equal to *one-half* of the (limiting) NADH concentration. Under the condition [NADH] > [E] > [NDMA], however, the disappearance of NDMA occurs *via a single* (apparent first-order) *kinetic process*. The rate of this transformation has a first-order dependence on the NADH-saturated enzyme-site concentration (over the accessible enzyme concentration range 4×10^{-7}–2×10^{-5} N). The assumed specific rate for this process is calculated to be 2×10^7 M^{-1} sec^{-1}. When both [NADH] and [NDMA] ≫ [E], a presteady-state "burst" reaction results in the disappearance of NDMA in an amount equivalent to *one-half* of the (limiting) concentration of enzyme sites. A comparison of the rate of NADH disappearance (at 330 nm) with the rate of NDMA disappearance (at 440 nm) under a variety of conditions demonstrates, by the nonequivalence of the two processes, an obligatory metastable intermediate. The intermediate can be formed in amounts in excess of the active-site concentration, and thus it can be released from the enzyme into free solution as a complex formed from the reactants.

A necessary condition for the comprehension of enzyme catalysis is a detailed knowledge about the transformation of reactant to product at the active site. Such knowledge includes the chemistry of any intermediates which may intervene.

In systems where a stable intermediate cannot be trapped, it has been informative to examine the transformation by combining uv–visible spectrophotometry or fluorometry with rapid kinetic techniques. In kinetic studies using the rapid-mixing stopped-flow spectrophotometer, it is useful to work under conditions of [E] > [S] (*i.e.*, the transient state). Frequently reaction is limited to a single turnover of sites under these conditions, and as a consequence, the kinetic behavior is simplified. Furthermore, these conditions favor the observation and chemical identification of individual steps in the catalytic mechanism.

In the present study, the intense chromophore, I, *p*-nitroso-*N*,*N*-dimethylaniline (NDMA), is introduced as an aldehyde substrate–analog for horse liver alcohol dehydrogenase (LADH). The use of NDMA as a substrate–analog has the advan-

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[1] Abbreviations used are: NDMA, *p*-nitroso-*N*,*N*-dimethylaniline; LADH, horse liver alcohol dehydrogenase; NADH, reduced nicotinamide–adenine dinucleotide.